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Volume 3 Issue 1

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SAN LUIS OBISPO

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For the young men and women in the sciences who have questions and the tenacity to discover the answers.

Table of contents

A NOTE FROM

THE EDITOR-IN-CHIEF

Dear Readers of Symposium:

As Editor-in-chief, I have learned a lot from planning the third issue of *Symposium*. Many of the students that do research in the College of Science and Mathematics have been working on their projects for a long time and are dedicated to their projects. Being able to showcase these projects is truly an honor.

For the second year in a row, we were fortunate enough to receive funding from the Cal Poly Instructionally Related Activities (IRA) program in addition to funding from the Baker & Koob Endowment. We are grateful to be funded by these two great programs as they have provided the journal with freedom and resources to form an excellent edition and have enabled us to further expand *Symposium*. Receiving grant money from such prestigious programs reaffirms how important it is to have a student-run journal that presents student research.

Our executive team is comprised of students from both the College of Liberal Arts and the College of Science and Math. From this issue specifically, we worked with students from English, Physics, Kinesiology, and Biology. Their talents ensure that the papers featured in the journal meet the standards of a professional publication, are easy to read, and are engaging to the reader. It has been a pleasure leading such a diverse group of students.

Parisa Mokhtari
Editor-in-Chief, 2015-201

SYMPOSIUM

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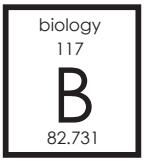
Research

Articles



Growth impacts of marine microplastics on the California mussel *Mytilus californianus*

A Research Proposal

by Andrea M. Fieber

Introduction

Background

As the global economy has expanded and intensified, the use of plastic materials in virtually every facet of life has become commonplace. Around the world, petroleum products take the form of water bottles, fishing lines, Styrofoam packaging and countless other single-use items, which are discarded and may easily find their way into the ocean through vectors such as storm water channels, urban wastewater discharge or direct dumping (Barnes et al., 2009; Carson et al., 2013). Plastic comprises ten percent of municipal solid wastes (Barnes et al., 2009), and approximately 12.7 million metric tons (mmt) of plastic are estimated to be added to the world's oceans annually as this refuse is intentionally or accidentally released into the environment (Jambeck et al., 2015). Plastics have been detected in all seas and oceans around the world, with the highest measurement recorded at 7,290 items per hectare (Barnes et al., 2009). Pollution monitoring and prevention methods are currently sparse and unreliable, allowing the problem to worsen until improved and enforceable mitigation is developed (Kershaw et al., 2011). With insufficient pollution controls in place, plastic debris continue to enter the oceans at the expense of marine ecological health.

Biological impacts of plastic pollution are widespread and appear to affect

organisms of all trophic levels and habitat types (Andrady, 2011). Seabirds (Mallory, 2008), sea turtles (Mascarehnas, 2004) and large marine mammals (Walker and Coe, 1989) are susceptible to entanglement or "ghost fishing" by discarded fishing nets and frequently ingest floating plastic debris. Twenty-six species of toothed whales, manatees and seals are known to consume plastic pieces (NOAA, 2014), and necropsies on beached sperm whales in Germany in January 2016 revealed plastic debris in 13 of the 29 animals' stomachs (Hoare, 2016). Thirty-five percent of seabirds are affected by plastic ingestion (Allsop et al., 2006) and albatrosses on Midway Atoll, a small wildlife refuge more than 2000 miles offshore in the center of the Northern Pacific Gyre, are often found deceased with guts full of plastic bottle caps and cigarette lighters (Jordan, 2011). The effects of marine plastics have been documented in over 660 species worldwide and can cause inflammation, blockages and lacerations within the gastrointestinal tract, frequently resulting in extreme distress or death of the animal (Oros et al., 2005). Plastic pollution has become a chronic source of physiological stress for numerous phyla, and no corner of the oceans has been untouched by its presence.

Marine plastics inhabit every oceanic stratum, often beginning as large floating items and drifting downward as they break into smaller pieces. After drifting on the surface of the water under direct sunlight, plastic polymers become photodegraded by ultraviolet radiation and begin to lose structural integrity, causing them to fragment (Singh, 2008). Compounds such as polyethylene and polypropylene, two common plastic constituents, cannot biodegrade and instead retain their inorganic properties while being mechanically divided and dispersed by wind and ocean currents (Reisser et al., 2013). Degradation progresses until plastics reach microscopic size; any piece measuring less than 5mm in diameter is considered a microplastic by the National Oceanic and Atmospheric Administration (NOAA, 2016). Adherence of microorganisms (biofilms) cause microplastics to lose buoyancy and settle on or within sediments (Graham and Thompson, 2009), where they may easily be ingested by benthic organisms.

Microplastics are known to be ingested and retained by a variety of marine invertebrates. Bivalves (Cauwenberghe and Janssen, 2014), crustaceans (Murray & Cowie, 2011), echinoderms (Graham & Thompson, 2009), copepods (Wilson, 1973) and zooplankton (Wright et al., 2013) have been observed consuming microplastics. Eighty-three percent of sampled lobsters (*Nephrops norvegicus*) from the Clyde Sea in Scotland were discovered with plastic filaments occupying or blocking their

gastrointestinal tracts (Murray & Cowie, 2011), and four species of deposit-feeding sea cucumbers have been shown to ingest more plastic particles than expected from sediments collected from the United States' east coast (Graham & Thompson, 2009). Deposit- and filter-feeders are susceptible to microplastic ingestion due to the analogousness of plastic particles to planktonic prey (Brillant & MacDonald, 2000). Organisms with ciliated feeding structures select their prey according to size rather than chemical makeup (Christaki et al., 1998), allowing microplastics within the allowable size range to be consumed as readily as food. As microplastics are undiscerningly ingested, harmful materials may be delivered to its consumer.

Microplastics are dangerous to the organisms that ingest them due to their transfer of toxic substances, resulting in bioaccumulation or direct poisoning. Drifting plastic particles, which are nonpolar, provide a medium for persistent organic pollutants (POPs) such as PCBs and DDTs (Wright et al., 2013), concentrating these substances onto ingestible vectors (Rios et al., 2007). As an animal attempts to digest a microplastic, these POPs are released in potentially fatal doses (Andrady, 2011). Additives such as brominated flame retardants, anti-microbial agents and colorings are found in many plastic items and may pose an additional threat to microplastics consumers (Thompson et al., 2009). Phthalate plasticizers and Bisphenol A (BPA) are common in mass-produced plastics, including PCV, and can easily leach from these materials (Thompson et al., 2009). These synthetic compounds have been shown to affect the reproduction and development of annelids, mollusks, crustaceans and fish (Oehlmann et al., 2009). Toxins that do not directly harm primary consumers may bioaccumulate along the food chain (Wright et al., 2013), and many of these substances have been shown to disrupt thyroid hormone regulation and induce cancer-causing imbalances in mammals (Talsness et al., 2009). Such impacts are compounded by the ability of microplastics to reduce the feeding efficiencies of many marine invertebrates.

Microplastic debris can drastically alter the effectiveness with which marine animals obtain nutrition both before and after it is ingested. Microscopic algae adhere to microplastics, restricting their photosynthetic productivity by reducing their intake of sunlight (Cole et al., 2011). Algae and plankton colonizing microplastic particles also become unavailable to organisms which sort and reject inorganic particles when feeding, compromising a major food source for such consumers (Wright et al., 2013). If ingested, microplastics occupying the digestive

tract can create blockages (Murray & Cowie, 2011), reducing the intake and digestion of necessary nutrients. Microplastics consumed by bivalves have even been shown to translocate into the animals' circulatory systems (Cauwenberghe & Janssen, 2014) where obstructions may also occur. Additionally, buildup of microplastics in the gut creates a false sense of satiation for the consumer as the stomach is filled with indigestible material, causing organisms to feed less frequently and on smaller meals (Pierce et al., 2004). Such behavior results in weight loss, a compromised immune system and eventual starvation (NOAA, 2014). However, it is unclear how these effects may have implications throughout the food web.

Marine bivalves such as mussels, oysters and clams are an important food source for many tertiary consumers, including humans. These filter-feeding organisms are commonly farmed in coastal waters around Europe, North America and China (Smaal, 1991) and are becoming an increasingly popular food source for developing nations (Naylor et al., 2000). Although mussel fisheries are expanding, it is unclear whether increased plastic pollution will have an effect on the yield of such operations. As outlined above, physiological stresses presented by microplastics may inhibit growth or kill mussels that are cultivated for consumption, and accumulated materials or toxins may render the organisms an unsafe food source. Microplastics may soon have far-reaching effects on worldwide fishing economies, but the magnitude and exact nature of the impact remains unknown.

Research Question

The California mussel *Mytilus californianus* is a common intertidal bivalve occurring along the entire western coast of North America (Bayne et al., 1976). Found in low-, mid- and high-intertidal zones, *M. californianus* is a sessile species that attaches to a substrate by the formation of byssal threads, which anchor the animal to protect it from wave action. Young organisms may increase in size by up to six millimeters per month, and growth slows as the animals reach sexual maturity and begin competing for space (Coe & Fox, 1944). As filter-feeders, they sweep water across gill sheets and use bands of cilia to capture, sort and ingest food particles; this strategy makes them more susceptible to microplastic ingestion than larger consumers which may graze or hunt their prey.

This study aims to determine the relationship between ambient microplastic concentration and growth rate of *M. californianus*, an economically important species that may experience increasingly direct interaction with microplastics throughout its Pacific range. *M. californianus* is commonly found on the Central Coast and easily sustained in a captive environment, making it a useful model organism. Rapid, observable growth and frequent use of congenerics *M. edulis* and *M. trossulus* as indicator species for pollutants (Hellou & Law, 2003) make *Mytilus* ideal for the study of microplastics' effects on ecological health.

Hypothesis

1. When subjected to microplastic ingestion, a threshold of tolerance exists for *M. californianus* for both a) the ability to maintain adequate body weight for normal systemic function and b) the ability to survive despite reduced fitness.

2. Individuals will experience progressively worsening health as microplastic concentrations increase, which may be due to insufficient nutrition, poisoning from microplastic POPs, compromised circulatory or immune systems or long-term stress from any combination of these factors. Such impacts may result in death for some individuals.

Research Design and Methods

Setup

Mytilus californianus, a commonly observed and commercially valuable marine bivalve native to the California coast, is used in this study. This organism takes up little space, is not highly sensitive to the conditions of a captive environment and is easily found and collected from nearby intertidal areas. Small (<30mm) mussels have been obtained, as they will grow more rapidly than larger individuals, producing more robust results in the 3-month data collection period. Upon collection, individuals have been sorted into six size classes to ensure consistency across treatments (see Table 1 below).

Each mussel has been placed in a clean glass beaker with 300mL of seawater filtered through 0.22-micron filters (Fisher Scientific, Waltham, MA)

and aerated by a small 2-watt aquarium pump (Tetra, Blacksburg, VA). Beakers are placed in a large incubator which maintains an interior temperature of 18.5°C and provides continuous light to the organisms. Mussels have been given two weeks to acclimate to the lab environment; they receive water changes every two to three days and a single daily feeding of 15 µL of commercial phytoplankton concentrate (Kent Marine PhytoPlex, Franklin, WI).

Treatments

Three experimental treatments are utilized for the study: low, moderate and high concentrations of microplastics. The "low" treatment receives 0.33 mg/L of microplastics to mimic concentrations observed in the Northern Pacific Gyre (Goldstein et al., 2012). The "moderate" treatment receives 675 mg/L to mimic conditions near a plastics production plant off the coast of Sweden (Noren & Naustvoll, 2010), which are among the highest recorded measurements in the world. The "high" treatment receives 1350 mg/L, or twice the amount of microplastic found near the Swedish coast, in a "worst-case-scenario" manipulation.

One animal from each size class has been assigned to one of the three experimental treatments or a control treatment, as summarized in Table 1. In each experimental treatment, a specific amount of microplastic is added to the animal's beaker during experimental periods; in the control group, animals will never interact with microplastics. Five-day experimental periods (Day 1 through Day 5 of each week) are followed by two-day "recovery" periods (Day 6 and Day 7 of each week). Glitter, an easily obtained and highly visible form of microplastic, is used for all treatments.

Table 1. 24 mussels have been distributed among six size classes, determined at the beginning of the study, and four treatments. A mussel identification code has been assigned to each organism to simplify data collection and analysis.

		Size class					
		i	*ii*	*iii*	*iv*	*v*	*vi*
Treatment	*Control*	1C	2C	3C	4C	5C	6C
	Low	1L	2L	3L	4L	5L	6L
	Moderate	1M	2M	3M	4M	5M	6M
	High	1H	2H	3H	4H	5H	6H

Measurements

Mussels are measured frequently for change in size from the previous data point. Data on both weight and length is gathered to assess growth and observe increases in both soft tissue mass and valve size, respectively. Measuring weight offers a more precise indication of the mussel's change in overall biomass, and observations of length are not subject to error from excess moisture on the organism; when considered together, these two parameters ensure an accurate dataset.

Growth data is collected on Day 1 and Day 5 of each week during the study, thus capturing changes that occurred during both the experimental and recovery periods. Organisms are removed from their beakers and measured, from anterior tip to posterior tip of the valves, to the nearest millimeter with a small metal ruler. After mussels are air-dried and thoroughly wiped of any excess moisture, each individual is placed on a scale and weighed to the nearest milligram.

Results

Preliminary analysis of working datasets reveals a nonlinear relationship between average growth rates within each experimental treatment. Length measurements show little variation between data points, so weight measurements are used as the principle data in determining growth. As Figure 1 below shows, overall change in weight (on average across all size classes) after eight weeks of study demonstrates a disproportionally high growth rate among individuals in

the "high" treatment, despite the expectation of reduced growth. Such a result may be partly explained by Figure 2, which separates average change in weight by experimental (microplastic-laden seawater) periods and recovery (uncontaminated seawater) periods. Here, the "high" treatment experiences extreme weight loss followed by extreme growth, possibly indicating the presence of a growth-inducing stress response in these individuals

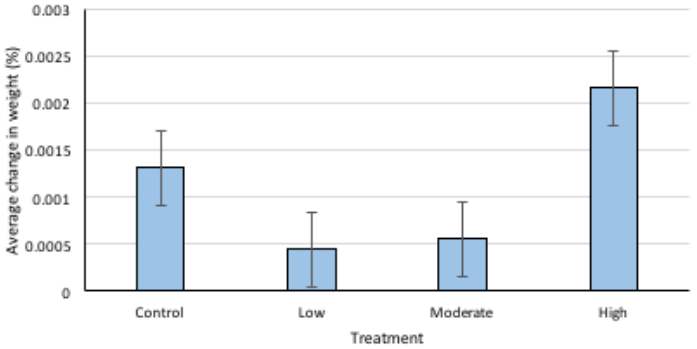


Figure 1. Overall changes in growth rate represent the predicted differences among treatments and are expected to develop a more consistent trend as the study continues. The error bars represent the standard error of the mean.

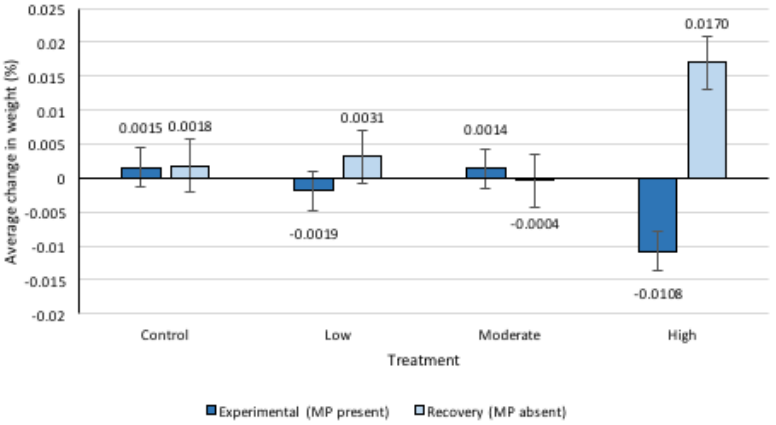


Figure 2. Data for "low" and "high" treatments exhibit growth rates that differ greatly according to the presence or absence of microplastics. Such a result may point to the presence of a physiological response to stress in M. californianus.

Continued data collection and analysis is expected to yield a more consistent trend throughout treatments, particularly within the "moderate" treatment, which currently has somewhat varied data. Existing anomalies may be explained by the small size of the dataset and may disappear as average changes in weight become distributed over a longer period of time. General data trends indicate support for the hypotheses of the study and are likely to remain consistent with these initial predictions.

Schedule

Experimental procedures are expected to begin in early February and continue until mid-May, allowing for 10-12 weeks of growth data collection. An estimated timeline is outlined below:

- Late January: Refine experimental design, conduct trial runs of logistical setup, acquire funding and necessary equipment
- Early February: Collect study organisms and begin treatments and data collection
- February, March, April, early May: Gather approximately 12 weeks of data, discarding deceased organisms as necessary
- May: Compile and analyze data, begin drafting final paper, develop discussion and poster for Cal Poly CSM Research Conference
- June: Finalize data analysis, complete paper for submission and potential publication

Budget

No funding is requested for equipment, as the use of an incubator and other lab materials is provided by the Cal Poly Center for Coastal Marine Sciences. Supplies include aquaculture materials such as aquarium hardware and food for the study organisms. Final results and analysis will be presented in poster form at the Western Society of Naturalists (WSN) conference in Monterey, CA in November 2016, and the project budget includes associated costs.

The anticipated budget for the project is outlined below:

Category	Estimated Cost
Personnel	
Undergraduate student ($10/hr, 3 hrs/wk)	$360.00
Equipment	$ 0.00
Supplies	$200.00
Travel	
WSN 2016 Registration	$145.00
Transportation (San Luis Obispo to/from Monterey, CA)	$ 50.00
Lodging	$125.00
Per diem costs ($40/day, 3 days)	$120.00
Total	$1000.00

Significance

Plastic has become an unavoidable ingredient of the world's oceans, though its effects on broader ecosystems are yet to be fully understood. Microplastic particles have been detected in ocean waters and sediments throughout the world (Wright et al., 2013), and their increased prevalence may deteriorate the health of marine ecosystems. Human societies, which are closely linked to marine resources, may face impacts as invertebrate stocks (an important source of nutrition and livelihood for many coastal communities) suffer as a result of plastic pollution. Hindered growth and development from accumulation of plastic particles and their associated toxins may drastically decrease yields of commercially farmed mollusks and crustaceans (Murray & Cowie, 2011), affecting supplies in a time of increasing global demand for ocean food products. The need for research-based understanding of this system is increasing, as effects of microplastics on growth rates remain unpredictable. This study aims to contribute to the growing body of knowledge on the impacts and need for prevention of marine microplastic pollution.

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Proteomic Response of Elephant Seal Pups, Mirounga angustriorstris, to Prolonged Fasting

by Melissa Voisinet, Maria Christina Vasuez, Cory Elowe, Dan Crocker, and Lars Tomanek

Introduction

Prolonged fasting is a stress commonly found in many genera of mammals. It is accompanied by shifts in metabolic pathways and likely affects the proteome of all tissues. An organism's ability to cope with periods of prolonged fasting is crucial for survival and is largely dictated by its life history. Many marine mammals have periods of fasting throughout their life; however, Northern Elephant Seals (*Mirounga Angustirostris*) (NES) in particular are subjected to periods of fasting up to 4 months depending on age and gender (Figure 1). Juvenile NES undergo an eight-week fast during the post-weaning period (Somo et al., 2015). During this period they transition from a terrestrial to mostly aquatic lifestyle (Reiter et al., 1978). This transition requires physiological developments adapted towards a greater capacity for diving and thermal regulation, which in turn require changes in energy metabolism and possibly anti-oxidant capacity (Le Boeuf & Richard, 1994).

In this study, we focused on the muscle tissue of NES pups to track the development of the proteome pre- and post-weaning over an eight-week period. The purpose of this study was to evaluate the proteomic changes in NES pups'

muscle throughout their post-weaning fast, a natural period in the pup's life, which is accompanied by a transition from a life on land to one in the ocean. Due to the increasing number of genome sequences and well-annotated expressed sequence tag (EST) libraries, proteomic analyses of non-model organisms have recently become possible. Although some work has been done on Phocid's survival during fasting (Vazquez-Medina et al., 2010; Tavoni et al., 2013; etc.), this is the first time that the proteome of a marine mammal has ever been analyzed. This will provide new insight on the adaptive capacity of marine mammals during this critical developmental stage.

Materials and Methods

Tissue Collection and Experimental Design

Tissue samples were taken from NES pups at a rookery in Año Nuevo, CA, USA (37°07'14.7"N 122°20'17.2"W) on February 14th and March 27th, 2015. Tissue samples were taken from skeletal muscle using a tissue punch and were flash-frozen in liquid nitrogen. After collection, the samples were transported to the Cal Poly Environmental Proteomics Lab on dry ice and kept at -80°C until homogenization. The animal care and use permits are held by Dr. Dan Crocker and approved by Sonoma State University and the University of California, Santa Cruz.

Protein Extraction

Muscle tissue samples were homogenized in glass homogenizers that were kept on ice. Supernatant was collected after centrifugation at 13,000g at room temperature. 400ul of protein from each sample was precipitated by adding four volumes of ice-cold acetone with 10% trichloroacetic acid and incubating at –20°C. Precipitated proteins were collected by centrifugation at 4°C. The pellets were washed with ice-cold acetone, centrifuged and then air-dried for 5 min. Protein samples were then re-suspended in 400 microliters of IPG rehydration buffer (Tomanek & Zuzow, 2010). Aliquots of the supernatant were stored in 2ml siliconized microcentrifuge tubes at –80°C. Protein concentrations were measured based on the BCA protein assay (Pierce, Rockford, IL, USA) using a microplate reader at an absorbance of 480nm.

Two-Dimensional Gel Electrophoresis

Immediately after resuspension, samples were combined with running buffer based on protein concentration, and then added to IPG strips (11 cm, pH range 3–10 non-linear; Bio-Rad, Hercules, CA, USA) (Tomanek & Zuzow, 2010). Isoelectric focusing began with 5h of passive rehydration, followed by 12h of active rehydration at 50V. Proteins were subsequently separated at 500V for 1h, 1000V for 1h and 8000 V for 2.5h. IPG strips were then frozen at –80°C until further use. To separate proteins according to the molecular mass, we first incubated IPG strips equilibration buffer with dithiothreitol (DTT) and then with iodoacetamide to reduce any disulfide bonds and quench the DTT (Tomanek & Zuzow, 2010). Strips were placed on top of 11.8% polyacrylamide gels and proteins were separated at 200V for 55min at 10°C (Criteron Dodeca; BioRad). Gels were immediately stained with colloidal Coomassie Blue (G-250) for 24h and then de-stained by washing repeatedly with Milli-Q water for 48h. Images of the gels were transferred to the computer using an Epson 1680 transparency scanner.

Spot Identification and Stastical Analysis

Delta 2D gel analysis software (version 3.6, Decodon, Greifswald, Germany) was used for image analysis and spot identification. Protein spots on all 2D gels were detected and warped to a reference gel from each time point. Subsequently, the reference gel from the post-weaned time point was warped to the reference gel from the pre-weaned time point. A master fused-image gel was then created by fusing all the gel images (Figure 2). This image contains all spots detected in any gel and at its maximal intensity. Using a spot detection tool, spots on the master gel were detected and possible artifacts were removed after visual inspection. We then generated a proteome map, which represents mean volumes for each spot. Spot boundaries from the master gel were then exported to all individual gels, thereby eliminating variability in spot detection and ensuring close spot matching. After background subtraction, protein spot volumes were normalized against the total spot volume of all proteins in a gel image. Normalized spot volumes for each time point were analyzed in Delta2D using a t-test with permutations ($p<0.05$).

Mass Spectrometry

Proteins that exhibited significantly different abundance between both time points were identified using mass spectrometry. Sample preparation for

the mass spectrometer was conducted as described by Tomanek and Zuzow (2010). Protein spots were briefly excised and de-stained twice. Proteins were subsequently digested with trypsin (Promega, Madison, WI, USA) overnight at 37°C. Trifluoroacetic acid (TFA) and acetonitrile were then used to extract digested proteins from the gel. Individual digested protein samples were mixed with matrix solution and spotted on a target plate (Bruker Daltonics Inc., Billerica, MA, USA). The spotted proteins were washed with 0.1% TFA and recrystallized using a mixture of acetone, ethanol and 0.1% TFA. A matrix-assisted laser desorption ionization tandem time-of-flight (MALDI-TOF-TOF) mass spectrometer (Ultraflex II; Bruker Daltonics Inc.) was used to obtain peptide mass fingerprints. A combination of peptide mass fingerprint (PMF or MS) and tandem mass spectra (MS/MS) data were searched against a metazoan database (NCBInr 20150404) using Mascot (version 3.1; Matrix Science Inc., Boston, MA, USA) to identify proteins. The molecular weight search (MOWSE) score that indicated a significant hit was set for >56 and the threshold p-value was set for <0.05 to confirm that the probability of a match was not a random event.

Results and Discussion

Protein gel image analysis showed 272 distinct protein spots found across both time points. Of these 272 protein spots, 44 (16%) significantly changed abundance between the two time points (permutation t-test, $P<0.05$) (Figure 3). Of the 44 significant proteins, 32 were identified using mass spectrometry. Of the 32 identified proteins, 7 proteins were identified as different isoforms that significantly changed between time points. Different isoforms of the same proteins may be present because of post-translational modifications that may change the molecular weight and isoelectric point of a protein. This will lead to the identification of the same protein as two or more different spots. Proteins were then grouped together based on general function.

Energy Metabolism

Six different proteins were identified which are known to be involved in energy metabolism. Two isoforms of glyceraldehyde dehydrogenase, two isoforms of fructose-bisphosphate aldolase A, mitochondrial aconitase, ES1 protein homolog and triosephosphate isomerase were all found to significantly change between the

two time points. All identified metabolic proteins were upregulated in pre-weaning pups and downregulated in post-weaning pups.

While fasting, NES pups continue to obtain energy through carbohydrates, lipids and, as a last resort, proteins. Carbohydrates are the preferred energy source because they are stored as glycogen in the liver and muscle; however, during fasting there are minimal carbohydrates available, so carbohydrate metabolism is downregulated during the post-weaning fast. All metabolic proteins found are enzymes that catalyze reactions of glycolysis. This supports that either glycolysis has decreased overall or that some of the metabolites of glycolysis from the earlier reactions are diverted to another pathway.

When carbohydrates are not available, one alternative pathway to glycolysis is gluconeogenesis. In this pathway, triglycerides are converted to glycerol and amino acids, while lactate is transformed to glucose. The majority of gluconeogenesis that occurs during a fast is from lipids, which are stored as a blubber layer in marine mammals. We saw no significant upregulation in lipolysis proteins, but we did find some lipolysis proteins that did not significantly change abundance, and were therefore potentially upregulated in both pre- and post- weaning pups. These lipolysis proteins were most likely upregulated in the pre-weaning time point due to the high fat content of the mother's milk.
Another potential alternative to glycolysis is the shunting of glucose-6-P towards the pentose phosphate pathway (PPP), which produces nucleotides that are needed during growth. The PPP may produce NADPH – used for producing fatty acids for adipose tissue. If the PPP synthesizes NADPH, it supports the hypothesis that the animal increases its fat deposits or invests into growth by producing RNA and DNA.

Cytoskeletal Structure

Proteins involved with cytoskeletal structure made up the largest fraction of identified proteins that exhibited altered abundances (~34%). Three isoforms of slow skeletal muscle troponin T, one isoform of myosin heavy polypeptide 7, one isoform of chain A structure of actin-bound WH2 domains of spire and the implication for filament nucleation, and alpha-actinin-2 isoform 1 all had high abundances in pre-weaning pups and were downregulated in post-weaning pups. Two isoforms of slow skeletal muscle troponin 2, one isoform of myosin heavy polypeptide 7, one isoform of Chain A structure of actin-bound WH2 domains

of spire, and the implication for filament nucleation showed low abundances in the pre-weaning pups and upregulation in post-weaning pups.

The up regulation and down regulation of cytoskeletal proteins show that there was a restructuring of skeletal muscle occurring during the post-weaning fast. The restructuring of muscle proteins are most likely a result of the pup's transition from a terrestrial to aquatic lifestyle, which requires them to use a new set of muscles. This is important because not only were the pups able to maintain muscle mass during their fast, but they were able to use energy to build new skeletal muscle as well.

Oxidative Stress and Proteostasis Proteins

Four different proteins were identified that are involved in oxidative stress. Two isoforms of glutathione S-transferase P-like, peroxiredoxin-6, alpha-crystallin B chain, heat shock cognate 71 kDa and three isoforms of heat shock proteins. One isoform of glutathione-S-transferase P-like, peroxiredoxin-6, alpha-crystallin B chain, heat shock cognate 71 kDa and all three isoforms of heat sock proteins were all upregulated in pre-weaning pups, and downregulated in post-weaning pups. The second isoform of glutathione-S-transferase P-like was downregulated in pre-weaning pups, and upregulated in post-weaning pups.

Our results suggest a restructuring of antioxidant proteins. This restructuring is most likely due to post-translational modifications. Studies have shown that carbonylation, a common type of post-translational modification, is often caused by oxidative stress (Dalle-Donne et al., 2006). We originally predicted that although both periods are associated with different types of physiological stress, the pre-weaning period would ultimately be associated with less physiological stress than the post-weaning period. The pre-weaning period is associated with the stress of growing and avoiding adult males, and the post-weaning period is associated with transitioning to an independent and aquatic lifestyle. The downregulation of many proteins associated with protein protection during oxidative stress suggests that the post-weaning period, although the sources of stress are different, may actually inflict more physiological stress on the animal as the pre-weaning period, which contradicts our original prediction regarding physiological stress caused by weaning.

Blood Proteins

Four proteins involved with blood significantly changed abundance

between the two time points. Two isoforms of myoglobin, hemoglobin subunit A, serotransferrin and serum albumin all significantly changed abundance between time points. Both isoforms of myoglobin, serotransferrin and serum albumin all increased in abundance in post-weaning pups, while hemoglobin subunit A decreased in abundance in post-weaning pups. Both myoglobin and hemoglobin are used by the animal as a means to store oxygen in the body. The increase in myoglobin is important because myoglobin has a higher affinity for oxygen than hemoglobin and is therefore a last resort for muscles after the oxygen that is stored in the hemoglobin has been used. This shows that NES pups are developing a tolerance to hypoxic situations, such as those experienced during foraging dives.

Importance and Future Research

Although there has been some research conducted on NES ability to survive fasting (Vazquez-Medina et al., 2010; Tavoni et al. 2013), this is the first time stress responses in NES due to prolonged fasting have been analyzed using proteomics. We suggest that future work include a comparative analysis of proteomic responses in NES between pups, adult females and adult males. Furthermore, comparing NES proteomic responses to closely related species might help give insight to the adaptive capacity of marine mammals during a critical developmental stage.

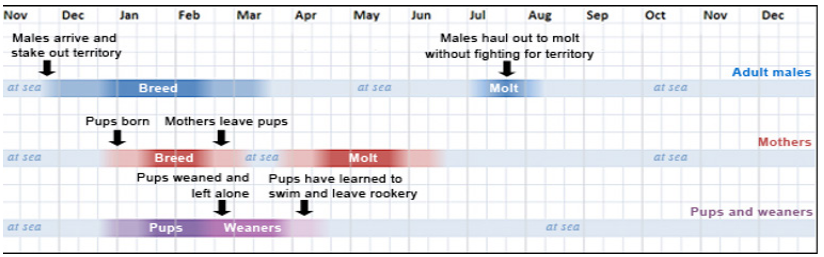

Figure 1: The annual life cycles of NES males, females and pups. Blue, red and purple shaded reagions indicate time spent on land. Light blue regions indicate time foraging at sea.

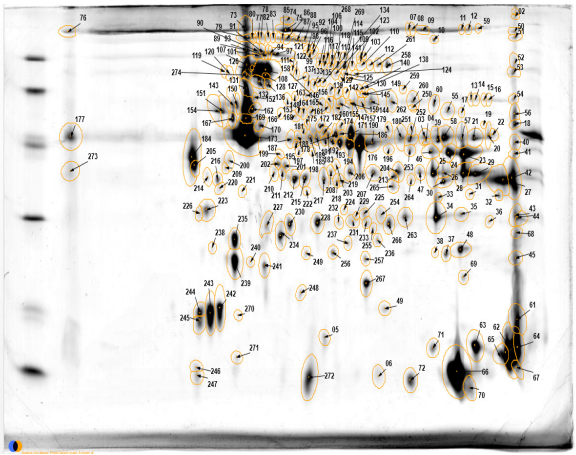

Figure 2: A fused gel image showing the 273 (44 sig.) individual protein spots detected. The proteome map represents the average normalized pixel volumes for each protein spot across all 12 gels. The number of spots that changed significantly between the two time points (pre- and post-weaning) was determined using a permutation t-test ($p < 0.05$). Isoelectric point ranges from pH 3-10 and molecular mass ranges from 14.4-97.4 kDa.

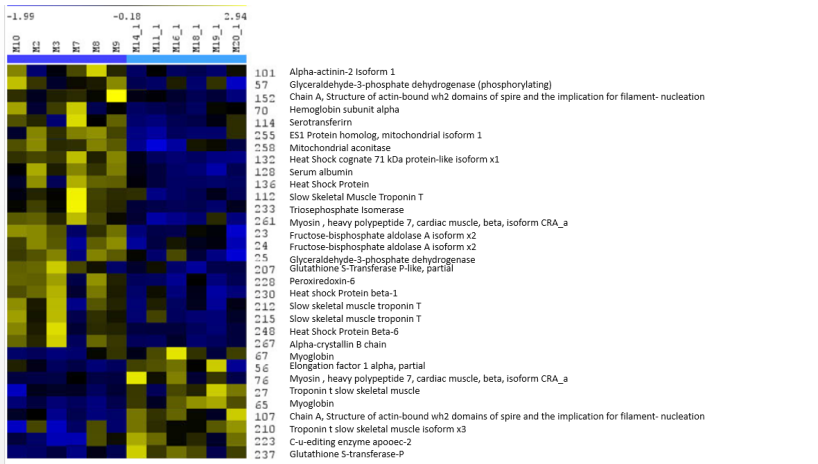

Figure 3: A heat map of the 32 identified proteins that significantly increased or decreased abundance between the two time points. The six columns on the left represent the six pre-weaning tissue samples, and the six columns of the right represent the six post-weaning samples. Each row represents a protein of different isoelectric point and molecular mass. Yellow represents a high abundance of protein, and blue represents a low abundance of protein. Spot numbers and protein identifications are listed to the right of the heat map.

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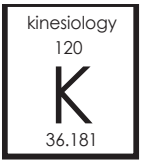

Effects of Branch Chain Amino Acid Supplementation on Energy Intake in Normal Weight and Overweight/Obese Individuals

by Jonathan Salcido, Tess Engel, Koa Cano, Pedro Angulo, Rebecca Brookes, Haley Kepler, Jason Lytle, Todd Hagobian

Abstract

Animal data suggests that branch chain amino acid (BCAA) supplementation, in particular leucine supplementation, suppresses energy intake. However, no study to date has evaluated the effects of these supplements on human energy intake. Thus, the purpose of this study was to determine the effects of BCAA and leucine supplementation on energy intake at a buffet meal and whether this differs by weight status. After an overnight fast, ten healthy, normal-weight adults (5M, 5F; BMI 21.5 ± 1.1 kg/m2; age 20.9 ± 0.60 yr) and nine overweight and obese adults (OW/OB; 6M, 3F males, BMI 27.7 ± 2.7 kg/m2; age 23.3 ± 2.6 yr) consumed one of three supplements on separate days in a block randomized, double-blinded design: 1) Placebo (calcium carbonate), 2) BCAA complex (5g leucine, 2.5g isoleucine, 2.5g valine) and 3) Leucine (10 g). Twenty minutes after total energy intake (kcal) at an open buffet meal was assessed using a repeated measures ANOVA. There was no significant (P>0.05) group by condition effect in normal-weight (617 ± 273, 570 ± 256, 683 ± 247 kcal, respectively) and OW/OB (823 ± 336, 766 ± 409, 831 ± 437, respectively) in energy intake at the buffet meal. In exploratory analyses in the normal-weight group alone, energy intake was significantly (P<0.05) reduced by 10% with the BCAA supplementation compared to the leucine and placebo supplementation. There was no significant difference (P>0.05) in OW/OB group alone between conditions. These data showed no overall effect of BCAA and leucine supplementation on energy intake. However, normal-weight participants appear to be more sensitive to the effects of BCAA supplementation on energy intake.

Introduction

Obesity is a growing trend in the United States, with reports that more than one-third of Americans are obese (Ogden et al., 2012). With the rise of obesity, there has been an increase in the incidence of Type-2 diabetes and cardiovascular disease as well. Many of these individuals attempt to lose weight via energy restriction or exercise, but the majority regain the lost weight. Dietary supplements are an alternative approach to induce weight loss and anecdotally show promise, but experimental data is lacking. In the past two decades, the supplement industry has grown with consumers, especially within the overweight/obese population attempting to make lifestyle changes. In recent animal studies, branch chain amino acid (BCAA) supplementation has been studied for the likelihood of decreasing appetite and altering metabolism favorably. These studies have shown promising results within mice models (Han & Lean, 2016). Previous studies have shown that leucine and isoleucine supplementation stimulates satiety hormones and impacts metabolism favorably (Lastya et al., 2014; Chen and Reimer, 2009). These studies indicate that branch chain amino acids can potentially affect essential hormones that help regulate hunger and satiety. Additionally, in another recent animal study, it was shown that leucine was important in reducing food intake when administering the supplement via intracerebroventricular infusion but not with oral intake (Zampieri et al., 2013).

The effects BCAA supplementation has on energy intake has not been studied in depth in humans, and thus, empirical data is lacking. The beneficial effects are yet unknown for either normal or OW/OB humans. Therefore, in this study the purpose was to determine the effects BCAA had on human adults and to measure whether oral intake of these supplements would inhibit energy intake at a buffet meal. We hypothesized that branch chain amino acids, particularly leucine, would have an affect on total energy intake at a buffet meal and thus alter the appetite of individuals.

Methods

Overview: The effects of branch chain amino acid and leucine supplementation on energy intake were assessed in normal weight and overweight/obese adult humans. A block randomized, cross-over and double-blinded design was

used in response to three conditions: 1) Placebo, 2) 10 grams of BCAA (5g leucine, 2.5g isoleucine, 2.5g valine) and 3) 10 grams of leucine. Energy intake at a buffet meal was assessed 20 minutes after each condition.

Subjects: Nineteen (12M, 7F) adults were recruited from California Polytechnic State University in San Luis Obispo, California by flyers and advertisements (Table 1). All participants were in good health with no known cardiovascular or metabolic diseases, as determined by a health-history questionnaire. Exclusion criteria included: psychological disorders (e.g. depression), cardiovascular disease, diabetes or any other metabolic diseases, significant weight loss in the previous 6 months (>5 kg), consumption of BCAA supplements or other supplements that could suppress appetite at the time of the study, illicit drug use, extreme dieting practices (e.g. Atkins, Paleo, etc.), history of bariatric surgery and if pregnant or expecting to become pregnant. This study was approved by the Human Subject Committee at California Polytechnic State University, and subjects provided verbal and written consent.

Table 1:Subject Characteristics

Variable	Normal Weight	Overweight/Obese Subject
Number of Subjects	N=10	N=9
Age	20.9 ± 0.6	23.3 ± 2.6
Body Weight (kg)	63.3 ± 7.9	85.1 ± 10.2
BMI (kg/m^2)	21.5 ± 1.1	27.7 ± 2.7

Note: Values are mean ± standard deviation.

Preliminary tests: Subjects completed a preliminary phone-screening questionnaire and, if qualified, they were asked to come to the testing center and a health-history and dietary questionnaire was administered. All subjects had their height in meters and weight in kilograms measured to determine preliminary body mass index.

Experimental Protocol: Subjects were asked to abstain from consuming alcohol, caffeine or performing physical activities (i.e. exercise, sports) twenty-four hours prior to each study visit to control for variables that may affect appetite or food intake. After an overnight fast (8-12 hours), subjects reported to the Human Performance Laboratory in the Kinesiology Department. Subject's

height and weight were measured, and an appetite and gastrointestinal distress questionnaire was assessed. In a block-randomized, cross-over and double-blinded fashion, subjects completed one of three supplement conditions: 1) Placebo (calcium supplement), 2) BCAA (5g leucine, 2.5g isoleucine, 2.5g valine) and 3) leucine alone (10 grams). After twenty minutes from the time of supplement consumption, the subjects were given another appetite and gastrointestinal distress questionnaire. Subjects were then given access to an open buffet meal for up to 30 minutes. Subjects were able to consume as much or as little of the food as they desired, and additional food was provided if asked. The subjects consumed food in isolation to prevent any distractions and minimize any influence on social or environmental factors. The buffet meal used in the current study mimicked previous studies and has been shown to be reproducible (Neary et al., 2004; Tepper et al., 2010; Hagobian et al., 2013). After the buffet meal, subjects completed another appetite and gastrointestinal distress questionnaire, and weight was assessed. Subjects returned for the following trial no more than one week later.

Buffet Meal: The buffet included a variety of carbohydrates, fat and protein and all items were present equally across all conditions. All items were either pre-weighed or served in their original container (e.g. drinks, cereals). Such items that were provided in the buffet included: cereals, milk (1% and whole), cereal bars, bagels, cream cheese, butter, bread (white and wheat), cheese, meat (ham, turkey, pastrami), fruit, vegetables, cookies, candy bars and juices. The food was prearranged in a designated room where a subject could eat in isolation. At the end of each meal, the food was measured by tallying the empty packages and reweighing any uneaten food. The weight of uneaten food was subtracted from the initial weight to determine the amount eaten by the subject. The main outcome was the total energy intake (kcal) consumed at the buffet meal.

Statistical Analysis: Energy intake at the buffet meal was assessed by group (normal-weight vs. OW/OB) and condition using a Repeated Measures ANOVA adjusting for baseline BMI, age and sex. In exploratory analysis, if no significant group x condition effect occurred, each group was assessed independently. A $P<0.05$ was considered significant and if differences occurred, a

Tukey's Post hoc was used.

Results

There was no significant (P>0.05) group x condition interaction in energy intake at the buffet meal (Figures 1 and 2). In other words, normal-weight and OW/OB groups did not differ in the amount of kcals consumed at the buffet meal. In exploratory analyses, however, the normal-weight group BCAA supplementation reduced energy intake at a buffet meal by 10% compared to placebo and leucine alone (P<0.05). In OW/OB group, there was no significant difference (P>0.05) between conditions.

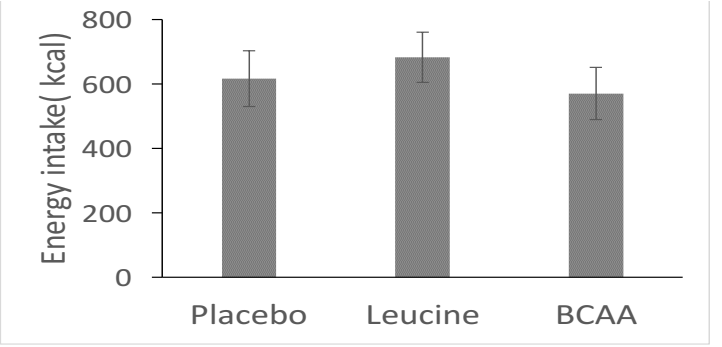

*Figure 1: Energy Intake at a Buffet Meal in response to each condition in normal-weight group. Values are mean ± standard error of mean. *BCAA supplementation significantly reduced energy intake by 10% compared to placebo and leucine supplementation. BCAA, branched chain amino acids.*

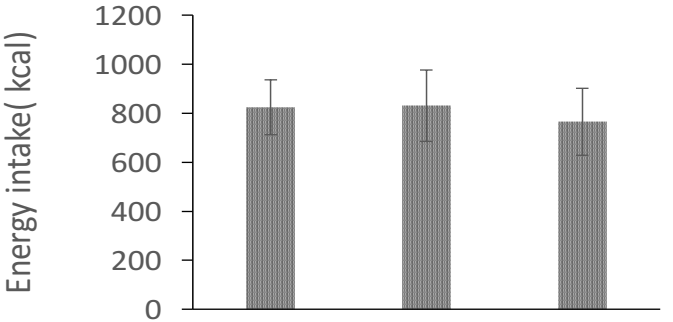

Figure 2: Energy Intake at a Buffet Meal in response to each condition in overweight/obese group. Values are mean ± standard error of mean. There was no significant difference between conditions. BCAA, branched chain amino acids.

Discussion

The goal of this study was to determine whether BCAA or leucine-only supplementation reduced energy intake at a buffet meal in normal-weight and overweight/obese adults. The main finding in this study was that there was no significant difference between groups in energy intake at the buffet meal in response to the three conditions. However, in exploratory analyses BCAA supplementation reduced energy intake in normal-weight but not overweight/obese. These data suggest normal-weight participants, compared to overweight/obese participants, appear to be more sensitive to the effects of BCAA supplementation on reducing energy intake.

In this current study, we did not observe any group x condition interaction on energy intake. These data are in contrast to previous animal data showing that BCAA or leucine supplementation reduces energy intake (Cota et al., 2006). In a recent study, leucine infused into rats' brains had an affect on the mTOR pathway located in the hypothalamus, which decreased food intake and weight in mice within 24 hours (Cota et al., 2006). Also, Zampieri et al. (2013) found that leucine was important in reducing food intake when administering the supplement via intracerebroventricular infusion but not with oral intake. In humans, empirical data evaluating the effects of BCAA and leucine supplementation on energy intake is lacking. Administering oral doses of BCAA to cancer patients with anorexia (not weight losing patients) may actually help safely increase appetite and bodyweight (Cangiano et al., 1996; Laviano et al., 2006). Nevertheless, there is debate as to whether that is always the instance, due to these studies being conducted in patients whose clinical symptoms cause muscular atrophy, and branch chain amino acids are shown to help with the production of neurotransmitters and protein synthesis. Thus, any increase in these supplements may ultimately have some neurochemical effects (Fernstrom, 2005).

In exploratory analyses, we found that BCAA supplementation reduced energy intake at a buffet meal in normal-weight but not overweight obese participants. In support of this difference, one epidemiological study showed that a higher BCAA diet in middle-aged subjects was associated with lower frequency of obesity in those countries (Qin et al., 2010). Experimental data is needed to confirm these results. Nonetheless, our data suggest that normal-weight, compared to overweight/obese, individuals appear to be more sensitive to the effects of BCAA supplementation on reducing energy intake.

A major strength of the current study was the block-randomized, double-blinded experimental study. But this study also has a few limitations. All subjects were college students; thus, the results of the current study may not

be generalizable to the general population. Additionally, we assessed the acute effects of these supplements on energy intake, and no study to date has assessed longer-term supplementation. Finally, we did not assess the effects of these supplements on appetite hormones (e.g. ghrelin, leptin, etc.) and thus, further studies are needed to evaluate any potential differences.

In summary, we noted no significant difference between normal-weight and overweight/obese groups in energy intake at a buffet meal in response to the three conditions. However, normal-weight, compared to overweight/obese, individuals appear to be more sensitive to the effects of BCAA supplementation on reducing energy intake. Future experimental studies are needed to confirm these findings.

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